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Lack of Protection In Vivo with Neutralizing Monoclonal Antibodies to Transmissible Gastroenteritis Virus

R.D. WESLEY[1], R.D. WOODS[1], I. CORREA[2] and L. ENJUANES[2]

[1]*National Animal Disease Center, USDA, ARS, P.O. Box 70, Ames, IA 50010 (U.S.A.)*
[2]*Centro de Biologia Molecular, C.S.I.C.-Universidad Antonoma, Canto Blanco, 28049 Madrid (Spain)*

ABSTRACT

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Monoclonal antibodies (Mabs) specific for the E1 and E2 surface glycoproteins of the transmissible gastroenteritis virus (TGEV) of swine were examined either alone or in combination to evaluate their potential value in protecting neonatal pigs against a lethal dose of TGEV. Cesarean-delivered colostrum-deprived (CDCD) piglets were given one pre-challenge dose of Mab and an equal dose of the same Mab at each successive feeding after challenge. In vivo challenge results demonstrated that neither Mabs given individually nor combinations of the Mabs were able to protect neonatal pigs against a lethal dose of TGEV. However, in parallel experiments, polyclonal antibodies from immune colostrum or serum were protective.

INTRODUCTION

The enveloped coronavirus, transmissible gastroenteritis virus (TGEV), contains surface E1-matrix and E2-peplomer glycoproteins, and a phosphorylated nucleocapsid protein closely associated with the positive-stranded RNA genome (Garwes and Pocock, 1975; Brian et al., 1984; Hu et al., 1985). The large E2 protein protrudes as club-shaped projections from the virus surface and contains the neutralizing antigenic determinants (Garwes et al., 1978/79; Hu et al., 1985). The E1 glycoprotein is more deeply embedded in the lipid envelope and is the predominant surface protein of coronaviruses including TGEV (Sturman et al., 1980; Wesley and Woods, 1986). Competitive inhibition studies with monoclonal antibodies (Mabs) have revealed one dominant neutralizing site, Site A, on TGEV whereas neutralizing Mabs against one or

more non-site A domains occur at lower frequencies (Jimenez et al., 1986; Delmas et al., 1986; Correa et al., 1988). Woods et al. (1988) have shown that anti-E1 Mabs neutralize TGEV in the presence of guinea pig, rabbit and swine complement (C′).

TGEV is a leading cause of neonatal death in swine during the first 2 weeks post-parturition. Passive immunity from colostrum and post-colostral milk of immune sows is crucial in providing immediate protection for neonates against TGEV infection. Oral infection of the pregnant sow establishes high levels of secretory IgA (sIgA) in colostrum and post-colostral milk. Both the IgG and sIgA immunoglobulin fractions of colostrum and post-colostral milk from immune sows are protective when measured amounts are fed to susceptible piglets (Stone et al., 1977).

The purpose of this paper is to determine if murine Mabs that neutralize TGEV in cell culture also protect baby pigs. Identification of protective Mabs directed against a single epitope or a combination of epitopes is essential in the development of immunoprophylatic measures.

MATERIALS AND METHODS

Virus

Virulent pig-passed TGEV (Miller strain p439+) was kindly provided by Dr. Linda Saif (Ohio Agricultural Research and Development Center, Wooster, OH). A stock of challenge virus was prepared by orally infecting seven 3-day-old cesarean-delivered, colostrum-deprived (CDCD) piglets and harvesting the small intestine at the onset of diarrhea (24–40 h post-infection). The frozen small intestines were combined, homogenized in a Waring blender in cold 0.85% NaCl and filtered through sterile gauze. Homogenized supernatant (600 ml) was mixed with 400 ml fetal bovine serum and 1.5-ml aliquots were stored in liquid nitrogen. Thawed aliquots of the stock challenge virus had a plaque titer of 3×10^6 plaque-forming units (P.F.U.) ml^{-1} on swine testicular (ST) cells.

Housing and feeding

CDCD piglets were obtained from sows that were seronegative for TGEV neutralizing antibody by a plaque reduction assay on ST cells. The piglets were housed in individual plexiglass isolators in a room maintained at 35°C. These isolation chambers were kept under slight negative pressure to minimize contamination by atomized virus.

Cows milk was treated with β-propiolactone at a final concentration of 0.3%, aliquots were dispensed into sterile liter bottles and allowed to stand at room temperature at least overnight before use (Amtower and Calhoon, 1964). The

daily ration of β-propiolactone-treated cows milk was divided into three 60-ml feedings during the first 3 days and gradually increased to 100 ml per feed by Day 13.

Virus challenge

The appropriate antibody was supplemented in the morning feeding prior to challenge for those piglets that were to be continuously fed either Mab, colostrum or hyperimmune antiserum. To maximize the protective effect of antibody, the challenge dose was incubated in vitro (1 h at 37°C) with an excess of antibody as follows. For each experiment, 3 random aliquots of challenge virus were thawed, mixed and diluted 10-fold in PBS. The virus was then sonicated in two 20-s bursts in order to disrupt virus aggregates. The sonicated virus was further diluted in F-15 media (Gibco Laboratories, Grand Island, NY) supplemented with 2% fetal bovine serum to a final dilution of either 10^{-3} or 10^{-4}. The diluted virus (5 ml) was incubated (1 h at 37°C) with an equal volume of colostrum, hyperimmune antiserum or Mab either with or without 3% guinea pig serum as a source of 5.5 units of C'. The virus–antiserum, virus–colostrum and virus–Mab inocula were negative when titered on ST cells. Control animals received 5 ml of challenge virus incubated (1 h at 37°C) in an equal volume of F-15 media containing 2% fetal bovine serum. In lieu of an afternoon feeding, each experimental pig was given the challenge virus plus antibody mixture by stomach tube. In the evening, regular feedings were resumed on a 3 times per day schedule. Where appropriate, each feeding was supplemented with the following amounts of antibody: 20 ml colostrum [virus neutralizing (VN) titer = 1:980], 4 ml antiserum (VN titer = 1:1280), 2.9 ml Mab 1A6 (VN titer = 1:59 700 in the presence of 5 units of C'), 3.1 ml Mab 4F6 (VN titer = 1:4000) and 4.5 ml Mab 5D5 (VN titer = 1:8000). The pigs were observed for the onset of clinical signs and death during the 10 days following challenge. The VN titers were determined by a 50% plaque-reduction assay (Woods et al., 1988).

Monoclonal antibodies, colostrum and hyperimmune serum

Mabs directed against TGEV E1 (Mab 1A6, isotype IgG 2b) and E2 (Mab 4F6, isotype IgG 2b; Mab 5D5, isotype IgG 2a) structural proteins were prepared essentially as described by van Deusen and Whetstone (1981). Primary hybridomas that were secreting antibody were cloned twice and administered intraperitoneally to pristine-primed Balb/c mice. The resultant ascites fluids (~300 ml per Mab) were collected, pooled, clarified (2000×g for 15 min) and heat inactivated (30 min, 56°C) before use.

To obtain colostral whey, a pregnant sow which was seronegative for TGEV neutralizing antibody was inoculated orally/nasally, 6 weeks prior to farrow-

ing, with a single 30-ml dose of intestinal homogenate containing Miller virus. No fever or diarrhea occurred following inoculation, however, the sow was off feed for a few days. Colostrum (500 ml) was collected at the time of farrowing and again (600 ml) ~12 h after farrowing. For milk letdown, 20 units of oxytocin (TechAmerica, Elwood, KS) was given intravenously. The colostrum was centrifuged ($1600 \times g$, 20 min) and the whey stored in aliquots at $-20°C$.

TGE immune serum, kindly provided by Dr. A.W. McClurkin (National Animal Disease Center, Ames, IA), was prepared as follows. Two 30-ml doses of virulent TGEV were administered to a pregnant sow 7 and 3 weeks prior to farrowing. Each dose, given intramuscularly, consisted of 2 parts sucrose gradient-purified virus and 1 part adjuvant (9 parts mineral oil and 1 part Arlacel A, Sigma Chemical Co., St. Louis, MO). On the second day post-farrowing, each piglet was exposed to ~10^4 pig-infectious doses of TGEV as intestinal homogenate. No signs of disease were observed in either the pigs or the sow. Fourteen days later, the piglets were removed; the sow was given 2 intravenous doses, 3 weeks apart, of 25 ml of virus without adjuvant. Seven days after the last injection, the sow was anesthetized and bled.

Radioimmunoprecipitation assay

The specificity of Mabs 5D5, 4F6 and 1A6 was determined by a radioimmunoprecipitation procedure previously described by Wesley and Woods (1986). Each Mab was tested with both a ^{35}S-methionine-labeled TGEV-infected ST cell lysate and with labeled mock lysate as a control.

Competitive radioimmunoassay

Mabs were purified by high pressure liquid chromatography (HPLC) from ascitic fluids (Deschamps et al., 1985), ^{125}I-labeled (Greenwood et al., 1963) and the inhibition of their binding to purified virus by unlabeled Mabs was studied by radioimmunoassay (RIA) as described by Correa et al. (1988). The studies were performed with 5 standard Mabs, representative of the 4 antigenic sites of E2 protein: 1D.B3 and 6A.C3, Site A; 1B.H11, Site B; 5B.H1, Site C; 1D.G3, Site D.

RESULTS

Standard virus challenge

In order to ensure a uniform virus challenge dose, aliquots of pig-propagated virulent TGEV (Miller strain) were stored in liquid nitrogen. For each experiment, 3 random aliquots were thawed, mixed and sonicated prior to dilution in tissue culture medium. The titer of the challenge virus, assayed on ST cells, was 3×10^6 P.F.U. ml^{-1}. To determine the piglet LD$_{50}$ of the challenge virus,

TABLE 1

Titration of the standard challenge virus (Miller strain) in piglets

Virus dilution	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
No. of survivors	0/2	0/3	0/3	1/4	3/4	2/2

$LD_{50} = 10^{5.5}$, estimated by the method of Reed and Muench (1938).

3-day-old CDCD piglets were inoculated via stomach tube with 5 ml of 10-fold serial dilutions of challenge virus and the number of survivors recorded after 10 days (Table 1). The piglet LD_{50} was estimated to be $10^{5.5}$. The 2 challenge doses selected had an LD_{50} of 50 at a 10^{-4} dilution (1500 P.F.U.) or 500 LD_{50} at a 10^{-3} dilution. The challenge doses were purposely kept low so that there was a vast antibody surplus during the in vitro incubation with virus and so as not to overwhelm any positive effects produced by effective neutralizing TGEV antibodies.

Protein and epitope specificity of TGEV neutralizing Mabs

Three different TGEV neutralizing Mabs were selected to test for their ability to protect baby pigs in vivo. Two Mabs, 4F6 and 5D5, were directed against the E2-peplomer protein as shown in Fig. 1. Mab 1A6 was directed against the
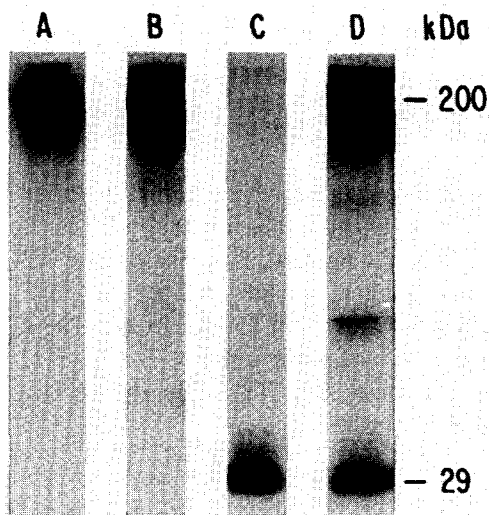

Fig. 1. Protein specificity of monoclonal antibodies. (A) Immunoprecipitation of ^{35}S-methionine-labeled, TGEV-infected cell lysate with anti-E2 Mab 5D5; (B) immunoprecipitation with anti-E2 Mab 4F6; (C) immunoprecipitation with anti-E1 Mab 1A6; (D) E1 and E2 antigens immunoprecipitated with a pig convalescent anti-TGEV serum.
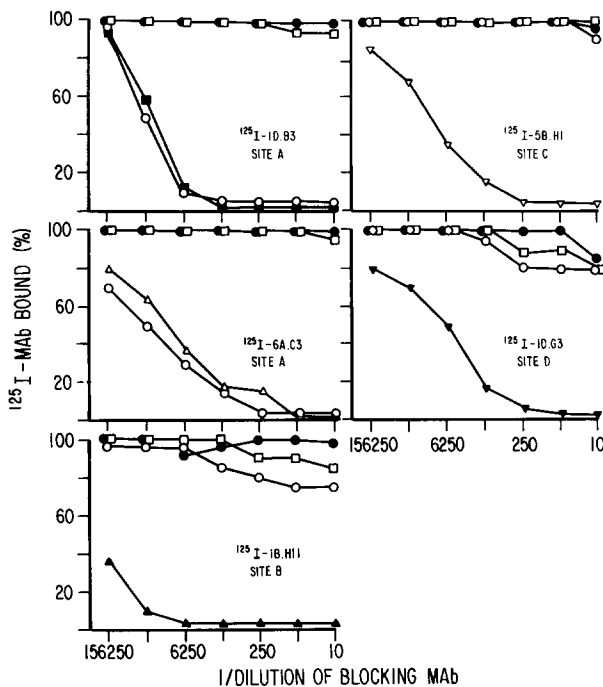
Fig. 2. Competitive binding of E1-specific Mab 1A6 and E2-specific Mabs 4F6 and 5D5 with ^{125}I-labeled Mabs that define E2 antigenic sites A, B, C and D. In a solid-phase RIA, a dilution series of unlabeled Mab 5D5 (●), 4F6 (○) and IA6 (□) competed for viral binding sites with Site A-specific Mabs 1D,B3 (■) and 6A.C3 (△), Site B Mab 1B.H11 (▲), Site C Mab 5B.H1 (▽) and Site D Mab 1D.G3 (▼). Each of the ^{125}I-labeled Mabs also was competed with unlabeled homologous Mab.

E1-matrix protein (Fig. 1) and neutralized TGEV in the presence of complement (Woods et al., 1987).

To determine the epitope specificity, one-way competitive inhibition experiments were carried out. Figure 2 shows that the neutralizing Mab 4F6 is directed against antigenic site A, the predominant neutralizing site on the E2-peplomer protein (Correa et al., 1988). In contrast, neither anti-E2 Mab 5D5 nor anti-E1 Mab 1A6 inhibited the binding of Mabs that Correa et al. (1988) have shown to be representative of E2-specific antigenic sites A, B, C and D. These results suggest that Mabs 4F6 and 5D5 neutralize TGEV by interacting at different epitopes on the E2-peplomer protein.

Lack of passive protection with Mabs

Anti-E1 ascitic fluid containing Mab 1A6 efficiently neutralized TGEV in vitro only in the presence of C' (Woods et al., 1987). This complement-depen-

TABLE 2

Lack of passive protection with neutralizing Mabs

Group	Treatment[a]	Supplemental Mab[b] at each feed	Number of survivors		Number with clinical signs	
			50 LD_{50}	500 LD_{50}	50 LD_{50}	500 LD_{50}
I	1A6	None	0/3	1/3	3/3	3/3
II	1A6+C'	None	1/3	0/3	3/3	3/3
III	1A6+C'	2.9 ml Mab 1A6	1/3	ND	3/3	ND
IV	5D5	4.5 ml Mab 5D5	2/3	0/2	3/3	2/2
V	4F6+1A6+C'	3.1 ml Mab 4F6	1/3	ND	3/3	ND
Controls	Diluent only	None	1/6	0/3	6/6	3/3

[a]Virulent virus and Mab or F-15 diluent incubated (1 h, 37°C) in vitro prior to baby pig challenge. Guinea pig serum at a final concentration of 3% was used as the source of 5.5 units of C'.
[b]The amount and type of Mab supplemented in the feeding before challenge and at each feeding after challenge for the 10-day observation period.

dent neutralizing mechanism could be irreversible by causing holes to be produced in the virus envelope (virolysis) or by C' coating the outer surface of the virus. To determine the in vivo protective capacity of the complement-dependent mechanism of neutralization, Mab 1A6 was tested in piglets in different ways (Table 2). One group of pigs was challenged with virus incubated with Mab 1A6 alone and a second group challenged with virus treated with Mab 1A6 plus 5.5 units of complement (1A6+C'). The third group likewise received Mab 1A6 and 5.5 units of C'-treated virus and 2.9 ml of Mab 1A6 was fed 3 times per day for the duration of the experiment. The piglets receiving Mab 1A6-treated inoculum, either with or without C', were fully susceptible to infection even though no virus was detected in the Mab 1A6+C' inoculum. Thus, even though tissue culture assays show virus neutralization by Mab 1A6+C', piglets remained fully susceptible to this challenge, indicating that the 1A6+C' does not irreversibly neutralize all of the virulent virus.

E2-specific neutralizing Mab 5D5 was used alone (Group IV), or Mab 4F6 was used in combination with complement-dependent anti-E1 Mab 1A6 (Group V) to inactivate virus prior to challenge (Table 2). Although Mabs 4F6, 5D5 and (1A6+C') completely neutralized the inoculum in vitro, acute clinical signs of diarrhea, dehydration and depression developed in pigs receiving the Mab-treated virus. In the Mab 5D5 group, 2 of the 3 pigs survived challenge at 50 LD_{50}, showing marked clinical signs of emaciation and dehydration, but no Mab 5D5-treated pigs survived the 500 LD_{50} challenge dose.

Complement-dependent anti-E1 antibody 1A6 plus 5.5 units of C' and complement-independent anti-E2 antibody 4F6 were incubated simultaneously with the challenge virus (Group V, Table 2). Additionally, Mab 4F6 (3.1 ml) was supplemented at each feeding. The combined effects of E2-specific Mabs 4F6

and $(1A6 + C')$ at concentrations that neutralize TGEV in vitro, did not prevent morbidity or reduce mortality in piglets.

Passive protection with colostrum and TAGEV antiserum

To ensure that the feeding schedule and the environmental conditions within the piglet isolation chambers were satisfactory for survival, polyclonal antibody given as immune colostrum and hyperimmune antiserum were used instead of the neutralizing Mabs. Challenge virus ($50\ LD_{50}$) was incubated in vitro with either colostrum (Group I) or hyperimmune serum (Group II) before being administered by stomach tube (Table 3). These positive control animals received additional colostrum (20 ml) or antiserum (4 ml) at each feeding. Piglets receiving virus treated with either colostrum or immune serum remained healthy and alert during the 10-day post-challenge period. However, one piglet in the colostrum treatment group (Group I) died suddenly on Day 9 post-challenge. This pig showed no signs of diarrhea before it died. At necropsy, the intestine appeared normal and no TGEV was recovered from the intestinal contents suggesting that the pig died from causes other than TGEV infection.

Additional evidence suggested that the piglets in the immune serum group, Group II, were completely protected and were not infected by the challenge virus. At the end of the 10-day post-challenge observation period, these 3 piglets from Group II were combined in a single pen with 4 other surviving piglets that had shown clinical signs of TGE. During the week following common housing, only the 3 piglets from the immune serum group developed diarrhea and died. The other 4 convalescing piglets that survived the original challenge continued to recover and by post-challenge Day 24 had serum neutralization titers of 1:4500, 1:4500, 1:11 000 and 1:16 000. Apparently, one or more of the convalescing piglets were shedding virus at the time that the piglets were

TABLE 3

Passive protection with colostrum and hyperimmune serum

Group	Treatment[a]	Supplemental antibody at each feeding	Number of survivors		Number with clinical signs	
			$50\ LD_{50}$	$500\ LD_{50}$	$50\ LD_{50}$	$500\ LD_{50}$
I	Colostrum	20 ml colostrum	2/3[b]	ND	0/3	ND
II	Antiserum	4 ml antiserum	3/3	1/1	0/3	0/1
Controls	Diluent only	None	1/6	0/3	6/6	3/3

[a]Colostrum or antiserum incubated in vitro with virus (1 h, 37°C) prior to baby pig challenge.
[b]One pig was healthy until Day 8 post-challenge, when it became weak and lost its appetite. It was found dead the next morning and no TGEV was recovered from intestinal contents.

transferred into a single pen. Similar experiments with the colostrum control pigs (Group I) were not carried out.

DISCUSSION

Polyclonal antibodies and monoclonal antibodies (Mabs) that neutralize porcine TGE coronavirus in vitro are directed against the E2-peplomer glycoprotein (Garwes et al., 1978/79; Jimenez et al., 1986; Delmas et al., 1986). The E1-matrix glycoprotein is more abundant than the E2 glycoprotein in the virus envelope and E1-specific Mabs, 1A6, 4B11, 4G8 and 4F2, exhibit complement-dependent virus neutralization with VN titers that exceed those commonly associated with protective hyperimmune sera and E2-specific Mabs (Woods et al., 1987). We have tested our most potent neutralizing E2 Mabs and E1 Mab in the presence of C', to determine their capacity to passively protect piglets. The pigs were inoculated with virus treated with E1-specific Mab 1A6 and 5.5 units of C' or with an E2-specific Mab and additional Mab was fed in β-propiolactone-treated milk 3 times daily for the 10-day observation period. These studies have shown that the Mab+C' mixture which neutralizes TGEV in vitro did not protect baby pigs. In addition, neither the E1-specific Mab+C' mixture supplemented with E2-specific neutralizing Mab nor the E2-specific neutralizing Mab alone were protective for piglets, therefore, efficient virus neutralization in vitro does not correlate with the ability to protect piglets from TGEV challenge. Whether a fraction of the virulent virus escapes neutralization or the virus+antibody complex dissociates in the alimentary tract is currently under investigation.

Protection against TGE mortality in piglets is a result of neutralization of virus by antibody in the lumen of the alimentary tract (Haelterman, 1963). This is accomplished naturally by continuous immunoglobulin intake from the colostrum and milk of an immune sow. Piglets deprived of this immunoglobulin source are susceptible to experimental infection within 4 h after removal from the sow (Haelterman, 1963). Colostral IgG and sIgA from immune sows passively protect piglets (Abou-Youssef and Ristic, 1975; Bohl and Saif, 1975; Stone et al., 1977) although sIgA is superior because it persists in high titer in the post-colostral milk (Porter and Allen, 1972). Haelterman (1963) has shown that the antiserum from sows exposed to virulent TGEV also is sufficient to passively protect piglets and, in fact, one report suggests that antiserum administered to piglets with clinical signs of TGE altered the outcome of the disease (Noble, 1964). Therefore, if the antiserum or colostrum from an immune sow is fed regularly to piglets, then critical protective epitopes on virulent TGEV are neutralized in the lumen of the alimentary tract. We have repeated these results with CDCD piglets in isolation chambers fed 20-ml aliquots of colostrum per feeding or 4 ml of antiserum per feed. Three daily feedings protected the pigs if the challenge virus was first incubated (37°C, 1 h)

with antibody. Polyclonal serum completely blocked infection with TGEV because piglets fed hyperimmune antiserum and challenge virus were fully susceptible at 2 weeks of age when housed with convalescing pigs that were shedding TGEV. Since antibody against critical protective epitopes is present in immune colostrum and antiserum, then the appropriate Mab or combination of Mabs showing parallel degrees of passive protection would identify these critical epitopes.

Mouse ascitic fluids containing complement-dependent, E1-specific Mab 1A6 or complement-independent Mab 4F6 did not prevent morbidity or reduce mortality in piglets. E2-specific Mab 5D5 may have reduced mortality at the lower challenge dose (Table 2), but did not diminish observable clinical signs. One explanation for the lack of passive protection with murine Mabs may be that the virus–antibody complex dissociates after passing through the stomach of piglets and liberates infectious virus. However, porcine polyclonal antibodies in colostrum and in hyperimmune antiserum apparently do not dissociate because they are effective in inactivating TGEV and protecting piglets (Haelterman, 1963; Stone et al., 1977). These results suggest that perhaps the antigenic avidity of the murine Mabs used in this study was inadequate to survive passage through the piglet's digestive tract, whereas the avidity of the pig polyclonal antibody was sufficient.

A second explanation for the lack of protection with these neutralizing Mabs could be that a small fraction of virus may escape neutralization because the Mab is not specific for all virus variants present in the challenge virus inoculum. To limit the effect of virus variants that might escape neutralization, a minimal challenge dose, 50 or 500 LD_{50}, was used. No virus escape was detected by plaque assay after in vitro incubation of the challenge inoculum + Mab nor was there any significant difference in the onset of clinical disease for experimental piglets in which the inoculum was incubated with a Mab, and for control piglets. Additionally, in an attempt to minimize the effect of virus aggregation, the challenge virus was sonicated before in vitro incubation to expose all infectious particles to Mab.

A final explanation for the lack of passive protection may be that the Mabs in this study, although neutralizing in vitro, were not directed against viral epitopes critical for protection of the target cell in the piglet; i.e., viral neutralizing epitopes that cause plaque reduction on ST cells in vitro may differ from viral receptor sites involved in TGE viral entry and replication in swine intestinal enterocytes, thus leading to cell cytopathology and clinical disease in piglets.

Because the virus apparently neutralized by the Mabs, as assayed on ST cells, was not neutralized when assayed in vivo, additional studies are needed to determine if there may be important differences in TGEV replication on ST cells in vitro and in the natural target cells of the host-swine intestinal enterocytes.

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